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Studies of Metabolism in Tuberculous Lesions.

I. On the Mechanisms of N-acetyltyramine-formation From Tyramine by Mycobacterium tuberculosis.

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Introduction

The mechanisms of amino acid decomposition by bacilli or enzymes have already been studied by some investigators (Ehrlich,[1] Matsuda[2] and Hirai[3] etc.). Most of their works though were based on experiments of the action of B. coli. Recently, Shirai[7)~10] did research on the products from tyrosine in the culture media during the growth of tubercle bacilli and he found a new compound N-acetyltyramine,[11] a derivative of tyramine. This evidence is very interesting because it was generally believed that tubercle bacilli would produce some acids but no amine. Courmont et al.[5] reported that tubercle bacilli rather restrained the decomposition of the amine. Yamamura[6] also examined oxygen consumption in the culture media containing tyrosine during the growth of mycobacterium tuberculosis avium (Takeo) through the Warburg's manomater and assumed their decomposition. Shirai analysed the media after cultivation in which had contained tyrosine or tyramine at the outset and noticed tyrosol, tyramine, p-hydroxyphenyl-acetic acid other than N-acetyltyramine. The present author attempted to prove the results obtained first by Shirai and wanted to determine the formation of N-acetyltyramine and decomposition of tyramine and also their relationship with the decrease of glucose which seemed to be carbon-source for the new derivatives in the culture media.

If we could find any special compounds responsible for the action of tubercle bacilli in tuberculous lesions, it may present a new interesting problem in this scientific field. The results described in this report will, it is hoped, contribute something in this field.

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Experiments

1) Materials

1) Tyramine-HCl (p-hydroxyphenyl-ethyl-amine-HCl) (Prepared by prof. K. Hirai)
2) Mycobacterium tuberculosis avium (Takeo)
3) Sauton's synthetic medium

a) Asparagine	4.0	gm.
Potassium biphosphate	0.5	gm.
Ferric ammonium citrate	0.05	gm.
Magnesium sulfate	0.5	gm.
Aqua dest.	1000	c.c.
b) Glucose (20, 10, 5, 1 gm. in each experiment)		
c) Tyramine-HCl	1.0	gm.

2) Methods

(a) Preparation of Culture Medium and Inoculation

Glucose 20, 10, 5, 1 gm. in each medium and tyramine (1.0 gm) were mixed in Sauton medium (the above (3)), for the sterilization, it was heated at the pressure of 20 pounds' for about 15 minutes. For the inoculation, the bacilli were collected from the surface of the culture media. Bacillus which had been cultivated on the surface of the media was used for inoculation. The author observed that the sedimental lump of bacilli in the culture media of the vessels was probably due to their interrupted growth. Too high pressure in the sterilization procedure caused a dark-brown colour in the media. Such media were not used for the growth of bacilli.

(b) Duration of culture: 20 days at 37°C in each experiment.

(c) Treatment of culture-solution.

After cultivation, the medium was sterilized by heating and then the mycobacterium was filtrated through a filter paper, then dried and weighed. By such means, the conditions of the bacillary growth can be detected.

After the test of the acidity of the filtrate, the following extraction was performed.

(I) Extraction and Fractionation

As shown in Figure (I), filtrate solution (about 100 cc.) was evaporated and the residues were extracted with absolute alcohol by heating, and filtrated repeatedly through a filter paper until they became negative for Millon's reaction.

In each experiment, about 150 cc. of alcohol was used for this purpose. For the exclusion of alcohol, the filtrate was heated in the hot water-bath. The residue was dissolved again in a weak acidic water solution and mixed with ether over 72 hours and then divided into two fractions, the ether fraction and the water fraction.

The ether fraction was washed with a 10 % Na_2CO_3-solution of about 150 cc. (30~50 cc. each 3 time). After the separation of the ether fraction and the Na_2CO_3 fraction, the ether was excluded in a distiller by heating. The residue was dissolved in distilled water and examined for crystallization.

The other fraction of sodium carbonate solution was repeatedly extracted with ether for about 72 hours and separated again into the ether fraction and the sodium carbonate fraction. The ether fraction was treated in the same way as above mentioned. The sodium carborate fraction was neutralized and acidified with 10% HCl and extracted with ether for about 72 hours again.

By separation of this mixture, the author could obtain the ether fraction and HCl fluid fraction again. From this ether fraction, a crystal was obtained, after the exclusion of the ether. The HCl fluid fraction was tested after adjustment of the acidity and then discarded.

The water fraction was neutralized with 40% NaOH and alkalized with 10% Na_2CO_3 for about 100 cc. of solution and then extracted with ether for about 72 hours. In this case, the carbonate fraction and the ether fraction were obtained again. The ether fraction was washed with a 10 % HCl solution and separated into the ether fraction and HCl fluid fraction.

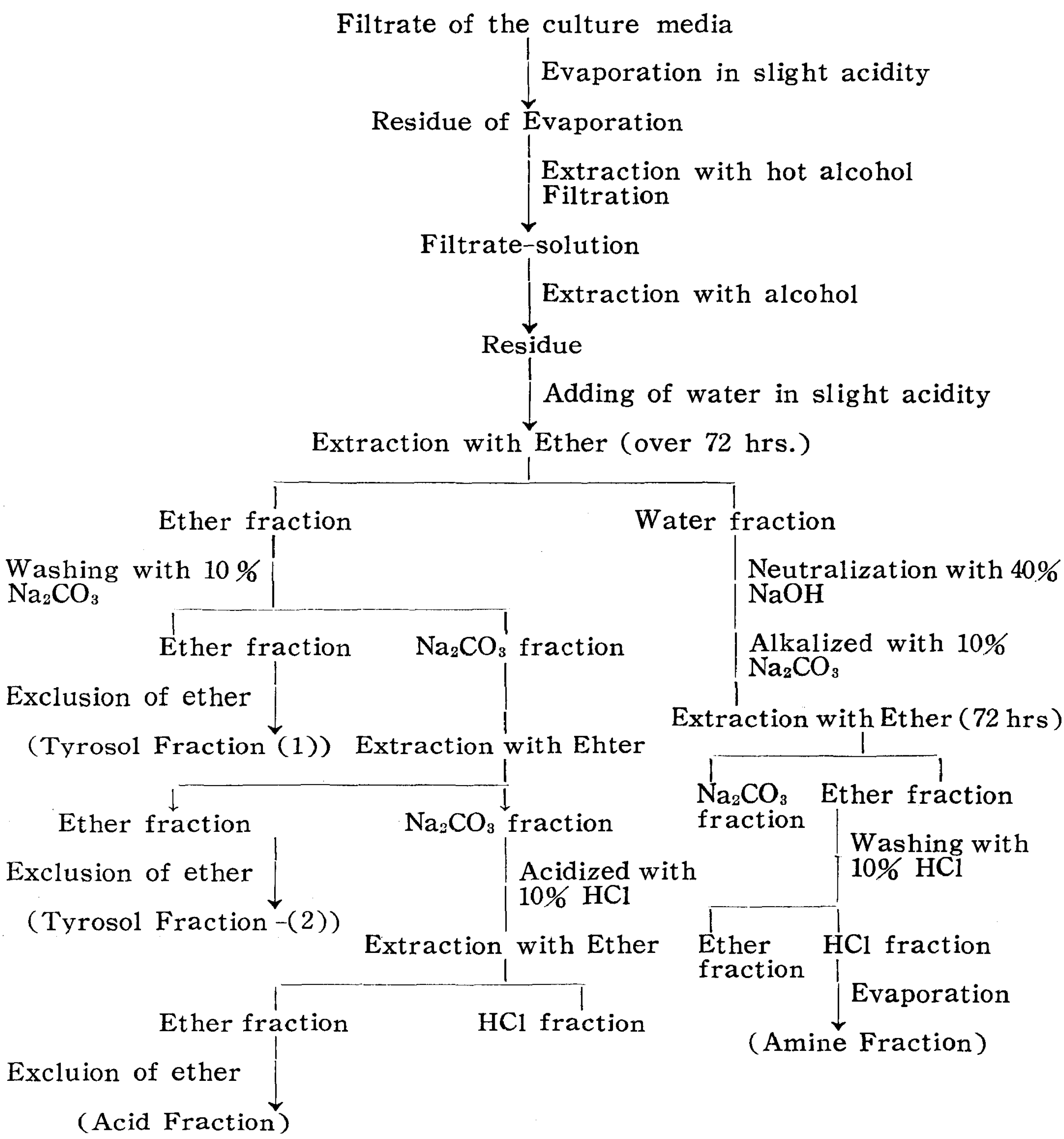
To make the solution negative for Millon's reaction, the ether fraction was distilled and the HCl fluid fraction was tested for crystallization. The carbonate fraction was discarded after confirmation of their negativity for Millon's test. (Note: each fraction was designated as described in Figure I. The numbers in round brackets in this Figure apply to Shirai's report[8].)

(2) **Crystallization**

The principal object of this examination is concerned with the formation of N-acetyltyramine, so the tyrosol fraction -(2) in which N-acetyltyramine might be formed was tested at first.

a) Tyrosol fraction -(2): In each experiment the culture media which contained 20, 10, 5 and 1 gm. of glucose was brown in appearance in various

Fig. 1 Methods of Fractionation.



degrees.

The substances responsible for the colour were examined on the slide glass and also extracted in the Erlenmeyer's flask with benzol of 50∼100cc 3∼5 times by heating and then filtrating. The filtrate was kept in the ice box. The white needle like crystal appeared. This crystal had a 128°∼129°C of melting point (M. P.), and, even when it was mixed with pure crystal of N-acetyl-tyramine, the M. P. did not fall. The author also ascertained it to be

N-acetyltyramine by the following paper chromatography.

b) Acid fraction : To obtain the crystal, the author evaporated ether from the fraction and dissolved the residue in distilled water in each experiment. But the brown needle like substance interrupted the experiment. It seemed to be same as in the case of tyrosol fraction. The author wanted to exclude this substance. And so, the solution was left on a open pottery clay-plate for exclusion for 2 days. The residue obtained by this procedure was dissolved in distilled water again and filtrated to re-crystallization. The necessary amount of crystal was obtained through by the experiment on the medium which contained 20 gm. of glucose.

On the contrary, in the case of all other experiments, only the oily substance remained in the clay-plate, so direct crystallization was attempted. The amounts of the crystals in this experiment were sufficient to measure their melting points. A minute quantity of the white crystal was negative for Millon's reaction.

A grey crystal was obtained in this experiment and it showed a intense positive reaction for Millon's test and a melting point of about 138°~140°C. The mixture test on this substance confirmed it to be pure crystal of P-hydroxypheyl-acetic acid. The author wished to do research on Rf. value by means of paper chromatography as in the other experiments.

c) Amine fraction: The light brown oily substance was found also in the amine fraction. From this fraction the author obtained a light grey crystal by re-crystallization as mentioned in the above.

This oily substance was too small in quantity to test even by using the clay-plate.

The author detected it only by means of paper chromatography. The M. P. of the crystals showed 266~267°C, 268°C and 268°C in the experiments which each contained 10, 5 and 1 gm. of glucose in the culture media. No change in M. P. was observed in this mixture and pure crystal of Tyramine-HCl.

Paper chomatographical analysis was also performed.

d) Tyrosol fraction-(1): Although some quantities of this substance could be obtained the fraction was usually too small, and it seemed impossible to test by the clay-plate method, there, for the author tested all these fractions by paper chromatography. The substance obtained from tyrosol fraction was a brown and oily.

Paper chromatography[14)~16)]

a) Solvent: N-Butanol : 2.5N NH_4OH 4 : 1

b) Paper : Tōyō filter paper No. 50, 51, 40 × 40cm, 2 × 49cm

c) Temperature: 25∼30°C (ordinary room temperature)

d) Visualizing reagent : a) Diazo reagent I, II. NH_4OH. 10% Na_2CO_3

b) Ninhydrine reagent. c) Millon reagent

The solvent used in this experiments was prepared by mixing n-Butanol and 2.5N solution of NH_4OH (the ratio 4 : 1). After shaking of this mixture, their superficial clear portions were used. Both No. 50 and 51 of Tōyō filter papers were used. The large paper was useful. The testing solution was dropped on the paper, a dot about 2∼3 m.m. diameter. As these experiments were performed in ordinary room temperature, it was sometimes 30°C.

In the visualizing procedure, 40cc. of Diazo reagent I. and 1cc of Diazo reagent II were mixed thoroughly and then spray on the completely dried paper. The paper was dried again and, then, spray by either ammonia solution or 10% solution of Na_2CO_3. Red colour could be appeared.

In the case of the test with ammoniak solution, the whole paper may became yellow in colour. So the Na_2CO_3 solution method seemed to be preferable to the former (NH_4OH) method.

In the Million reagent (diluted four times with distilled water) experiment, the spots were a dark gray colour. Consequently, the results obtained by this method were unsatisfactory.

Ninhydrin reagent was tested only for the detection of amino groups.

Results

The results (obtained) are shown in the figures and the tables.

Table 1 shows the quantity of the substances obtained from each fraction in the experiments above mentioned and the correlation is shown in Figure 2.

Table 2 shows the quantities and melting points of the substances in tyrosol fraction-(2) which were obtained in the first procedure in which N-acetyltyramine was already removed from the former substance.

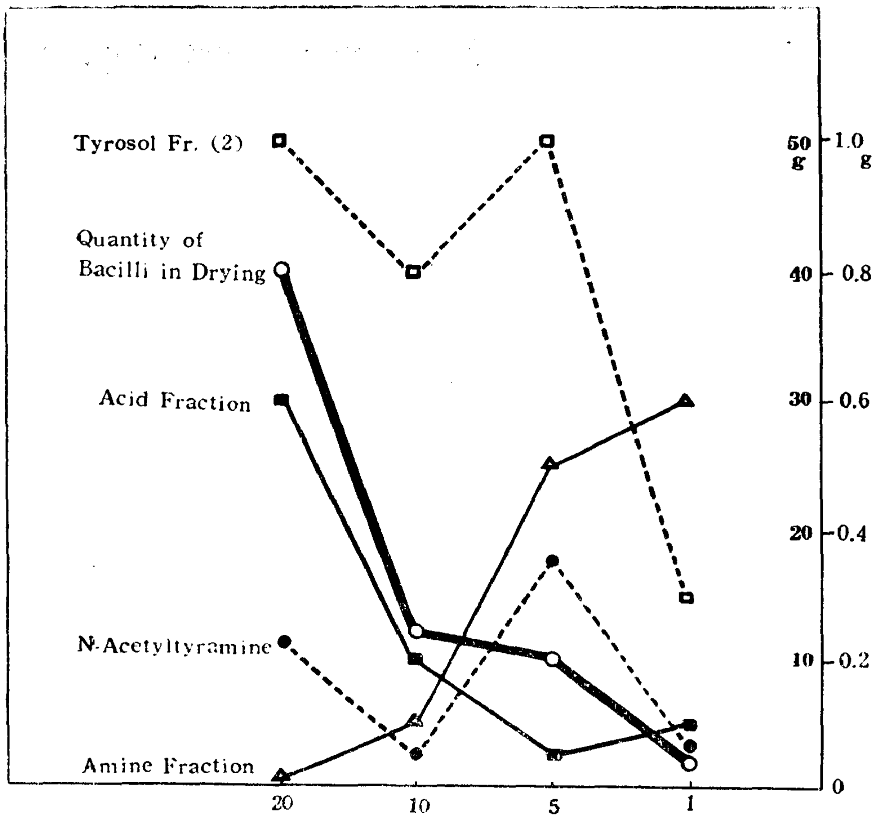
Table 3 shows the quantity obtained and M. P. of the tyramine.

Table 4 shows the concentration in each spot observed through the paper chromatogram. The sign of arrow (←) shows Rf value and indicates the relation of the two spots.

The arrow in tyrosol fraction-(1) indicates a shape like 8, although their boundary is indistinct, and definitely exists between 0.07 and 0.05 Rf. The sign of Ⓐ shows Rf value of pure crystal used as the control and Ⓑ indicates the results of the sample.

Figure 2 indicates the recovering tendency or the rate of increase of tyramine in the medium.

Fig. 2 The Tendency of Formation Substances.



Quantity of Glucose in Medium of 1 Liter.

Table 1

Quantity of Glucose in the Medium in 1 liter	20 gm.	10 gm.	5 gm.	1 gm.
Quantity of Tyramine-HCl in the Medium in 1 liter	1 gm.	1 gm.	1 gm.	1 gm.
Quantity of Bacilli in Drying	4 gm.	1.2 gm.	1.0 gm.	0.2 gm
Quantity of Acid Fraction	0.6 (0.4)gm.	0.2 gm.	0.05 (0.04) gm.	0.1 gm.
Quantity of Amine Fraction	(+)	0.1 gm.	0.5 (0.4)gm.	0.6 (0.45)gm.
Quantity of Tyrosol Fraction -(2)	1.0 gm.	0.8 gm.	1.0 gm.	0.3 gm.
Quantity of N-acetyltyramine	(0.22 gm) +x	(0.06 gm) +x	(0.35 gm.) +x	(0.06 gm.) +x

Table 2

Quantity of Glucose in the Medium in 1 liter	20 gm.	10 gm.	5 gm.	1 gm.
Quantity of the Oily Substance	1.0 gm.	0.8 gm.	1.0 gm.	0.3 gm.
Quantity of N-acetyltyramine	0.22 gm. +x	0.06 gm. +x	0.35 gm. +x	0.06 gm. +x
Number of Times of Extraction and Quantity of Benzol	100cc×3 50cc×2	50cc×1 100cc×3 50cc×1	100cc×3 50cc×1	100cc×3 50×2
Melting Point	128°C ~ 129°C	128.5°C ~ 129°C	128°C	128°C

Table 3

Quantity of Glucose in the Medium in 1 liter.	20 gm.	10 gm.	5 gm.	1 gm.
Quantity of Tyramine obtained from Amine Fr. (impure)	(+)	0.1 gm.	0.5 gm.	0.6 gm.
Quantity of crystal Tyramine, isolated from impure Tyramine		(+)	0.4 gm.	0.45gm.
Melting Points		266~267°C	268°C	268°C

Table 4

	Crystal \ Rf	0.87	0.71	0.14	0.09	0.07	0.05
Ⓑ	Tyrosol	+++					
	Tyramine-HCl		+++				
	P-hydroxyphenyl-acetic acid			+++			
	P-hydroxyphenyl-lactic acid				+++		
	N-acetyltyramine	+++					
Ⓐ	Tyrosal Fraction (1)						
	Quantity of Glucose in 1 liter of Medium 20 gm.	+				++ ←	+
	10 gm.	+				++ ←	+
	5 gm.	++				+++ ←	+
	1 gm.	±				± ←	+
	Tyrosol Fraction (2) (N-acetyltyramine)	+++					
	Amine Fraction		+++				

	Acid Fraction							
	Quantity of	20 gm.			+++ ←	++		
	Glucose in	10 gm.			± ←	+		
	1 liter of	5 gm.			++ ←	+		
	Medium	1 gm.			± ←	+		

Comments

As already described, many investigators thought that the tyrosine could not be digested by tubercle bacilli and also believed it counteracted to the bacilli, but Shirai[11] proved a possibility of the decomposition by tyramine as well as tyrosine by means of various culture media. Yamamura[6] also intimated the same idea. Hanke and Kössler[1] and Campbell[13] investigated histidine in their experiments with mycobacterium tuberculosis and they could not ascertain histamine in that culture media. Campbell[12] noted the fact that acetic acid was formed from alanine and, in the same manner, imidazol acetic acid from histidine. So, he asserted that the decarboxylic mechanism by tubercle bacilli might exist. He did not observe the formation of amines. Shirai and also the author at first believed the following to be the process.

Tyrosine→tyramine→tyrosol→para-hydroxyphenyl-acetic acid. As indicated in the results of this experiments, the author could obtain a minimal amount of tyrosol merely by the means of paper chromatography and also para-hydroxyphenyl-acetic acid, N-acetyltyramine and the other substance which was presumably para-hydroxyphenyl-lactic acid. Although para-hydroxyphenyl-lactic acid was obtained only in a small quantity, this fact seemed to be interested. The author should like to revise the cause of the metabolic change to the following instead of the above mentioned: tyrosol→para-hydroxyphenyl-lactic acid→para-hydroxyphenyl-acetic acid.

Tyrosol is, according to Shirai, hardly obtainable in his experiments and sometimes could not detected. As far as the present experiment is concerned, tyrosol was formed, and detected by the paper chromatography.

N-acetyltyramine and tyrosol showed the same Rf value by paper-chromatogram. It seemed to be impossible to detect these substances by such methods. It may be reasonable to suppose from their physicochemical characteristics that the substance isolated in tyrosol fraction-(1) might be tyrosol and the other substance in tyrosol fraction-(2) might be N-acetyltyramine. Decrease of the concentration of glucose in the culture media which is added as carbonsource to the products, will cause decreased growth of mycobacterium and also a decrease in the quantity of the products (Fig. 2). A relationship between the quantity of N-acetyltyramine which formed in the media and the growth of mycobacterium seemed to exist to a certain extent but no relationship was found between the quantity of N-acetyltyramine formation and the tyramine content as the nitrogen-source. The relationship of the concentration of glucose in the media and N-acetyltyramine is not clear as shown in Figure 2.

A relationship between the concentration of unchanged tyramine in the culture media and the amount of its acid fraction may eventually be established. When the large quantity of N-acetyltyrame was formed, para-hydroxyphenyl-lactic acid were scarcely found.

The author wished to explain the experimental results in such a way, the growth of mycobacterium will be controlled in such culture media by the conditions, especially the concentration of carbon-source, and in proportion to the growth of bacilli a large amount of N-acetyltyramine will be produced. As the carbon-source becomes deficient, the growth of bacilli depends chiefly upon the nitrogen-source and apparently accompanies the formation of acids. Such a relationship will be seen in Fig. 2. Occasionally, under too high pressure the preparation of culture media containing 1 per cent of glucose, under the clear media, changed to dark grey. Such coloured media was not suitable for the cultivation of mycobacterium. In certain circumstances, the bacilli will principally consume nitrogen compounds for their growth. This supposition is because the formation of N-acetyltyramine during the growth of bacilli decreased in the experiment on the culture medium in which a large quantity of glucose was added. This conception seems to agree with the results of Shirai's experiments in which a glycerin medium was used. Therefore, N-acetyltyramine will be obtained in the cases of experiments on media in which only small amounts of glucose as a carbon-source are contained.

As a matter of course, the acetylation process must be discussed. This process is perhaps not dependent on the simple chemical one, but the enzymes may play an important role. The participation of lipoid metabolism may be also expected. The unknown substances which appeared at Rf 0.07 and 0.05 by paper chromatography were also examined for further study.

Summary

Mycobacterium tuberculosis avium (Takeo) was cultivated in the culture media containing tyramine, a derivative of tyrosine, and also glucose as a

carbon-source for new compounds for a twenty day period. Disintegration of tyramine was noticed in this experiment.

In this process the following substances were obtained.

1) Tyrosol and para-hydroxyphenyl-lactic-acid like substances.

2) N-acetyltyramine and para-hydroxyphenyl-acetic acid.

3) Other unknown substances of Rf value 0.07 and 0.05 were detected by paper chromatogram.

It is interesting to note that the growing process of mycobacterium in which became slower in relation to the decrease of the concentration of glucose in the culture media.

By sterilizating the medium containing tyramine through high pressure for a certain period of time, a colour change of the medium to dark grey was sometimes noticed. Such a medium was not suitable for the cultivation of this bacillus.

The formation of N-acetyltyramine could be observed even in the low concentration (0.1%) of glucose as carbon-source in the media.

By decreasing the concentration of glucose in the culture media, a tendency of unchanged remainder of tyramine to increase in the media was noted.

It is rather interesting that the growth of mycobacterium diminished in accordance with a loss in quantity of glucose.

On sterilization of medium, through the process of high pressure for a incorrect time, we noticed that it was dark grey and the growth was not in a good condition.

Besides the above mentioned, it was observed that N-Acetyltyramine was formed even with 1 g. (0.1 %) of glucose as C-source.

It must be, once more reiterated that tyramine with a large quantity of glucose as carbon-source was almost spent and consequently the augmentation of existing tyramine still depended upon the decrease of glucose.

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